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Nitropyrene: DNA Binding and Adduct Formation in Respiratory Tissues

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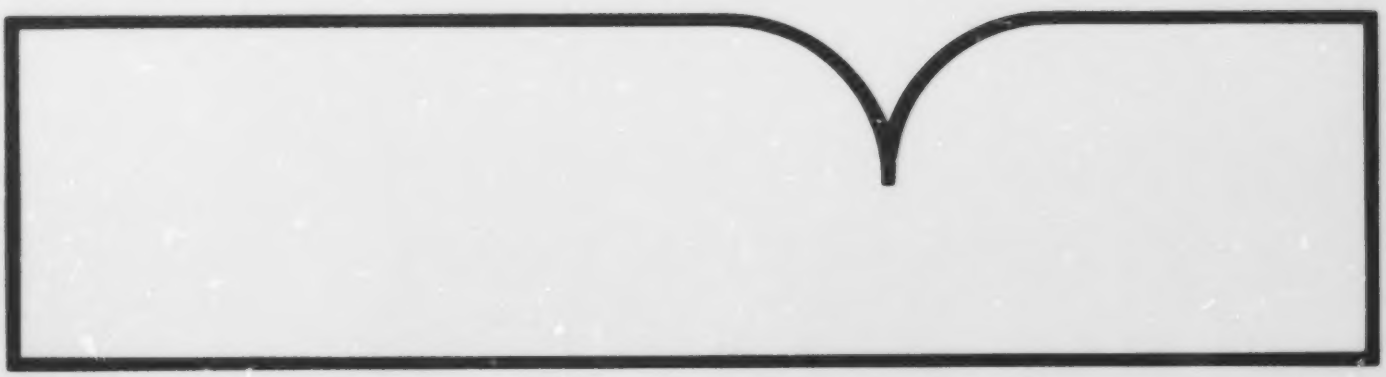

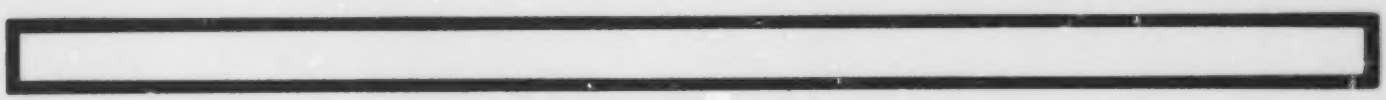

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16. ABSTRACT

Binding of 1-nitro(^{14}C)pyrene (NP) or its metabolites to cellular DNA and protein in cultures of rabbit alveolar macrophages, lung tissue and tracheal tissue was examined. DNA binding in tracheal tissue (136 ± 18.3 pmol NP/mg DNA) was four to five times the levels measured in either lung tissue (38 ± 9.4 pmol NP/mg DNA) or macrophages (26 ± 7.5-pmol NP/mg DNA). Adduct analysis of DNA isolated from lung tissue incubated with ^{3}H-1-nitropyrene in vitro resulted in the identification of 2-5% of the NP adducts as C8-deoxyguanosine 1-aminopyrene. NP was also bound to cellular protein in tracheal tissue and lung tissue, and at a lower level in macrophages. Co-cultivation of the macrophages with lung and tracheal tissue decreased the DNA binding in tracheal tissue by 45%. Following intratracheal instillation of diesel particles (5 mg) vapor-coated with ^{14}C-NP (380 ppm, 0.085 µCi/mg) particles into rats, 5 to 8% of the radioactivity remained in the lungs after 20 hours. Most of the diesel particles were also deposited in the lung.

Examination of DNA and protein binding in this tissue showed 5-12% of the pulmonary ^{14}C bound to protein and no detectable levels of ^{14}C bound to DNA.

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Nitropyrene: DNA Binding and Adduct Formation in Respiratory Tissues

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Binding of 1-nitro (^{14}C)pyrene (NP) or its metabolites to cellular DNA and protein in cultures of rabbit alveolar macrophages, lung tissue, and tracheal tissue was examined. DNA binding in tracheal tissue (136 ± 18.3 pmole NP/mg DNA) was four to five times the levels measured in either lung tissue (38 ± 9.4 pmole NP/mg DNA) or macrophages (26 ± 7.5 pmole NP/mg DNA). Adduct analysis of DNA isolated from lung tissue incubated with 1-nitro[H^3]pyrene *in vitro* resulted in the identification of 2 to 5% of the NP adducts as C8-deoxyguanosine 1-aminopyrene. NP was also bound to cellular protein in tracheal tissue and lung tissue, and at a lower level in macrophages. Cocultivation of the macrophages with lung and tracheal tissue decreased the DNA binding in tracheal tissue by 45%. Following intratracheal instillation of diesel particles (5 mg) vapor-coated with ^{14}C-NP (380 ppm, 0.085 μCi/mg) particles into rats, 5–8% of the radioactivity remained in the lungs after 20 hr. Most of the diesel particles were also deposited in the lung. Examination of DNA and protein binding in this tissue showed 5 to 12% of the pulmonary ^{14}C bound to protein and no detectable levels of ^{14}C bound to DNA.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) which pollute our atmosphere have been associated with the etiology of human cancer (*1*). Nitro-substituted PAHs (NO_2-PAH) have been found in extracts from diesel particles (*2–5*) and ambient air particulates (*6*). Many of these NO_2-PAH are mutagenic in mammalian cells (*7–10*) as well as in bacteria (*8–12*) and may, therefore, be a potential risk to human health.

Among these NO_2-PAH, 1-nitropyrene (1-NP) has been found to be a potent bacterial mutagen (*12*) and potentially an animal carcinogen (*13*). This compound has been identified and quantitated in diesel particle extracts (*3,4*) and may be responsible for up to 30% of the mutagenicity of these extracts (*4,14*). The bacterial mutagenicity of 1-NP is believed to result from reduction to reactive electrophiles which bind to DNA (*12,15*).

Howard and co-workers (*1*) have identified one DNA adduct formed by *Salmonella typhimurium* from the reduction of 1-NP as an *N*-(deoxyguanosin-8-yl)-1-aminopyrene, i.e., C8-deoxyguanosine-1-aminopyrene adduct (C8-dG-AP). They have also shown that some mammalian enzymes are capable of reducing 1-NP to form this same adduct. In addition, mammalian tissues are capable of oxidizing 1-NP both *in vivo* (*16*) and *in vitro* (*17,18*) to metabolites that are themselves mutagenic. We have shown in an earlier study (*19*) that rabbit pulmonary macrophages and tissues activated 1-NP to DNA-bound adducts.

In this work we evaluate DNA adducts resulting from the metabolism of 1-NP by mammalian cells and tissues to see whether and to what extent oxidative metabolism of 1-NP forms the C8-dG-AP adduct previously characterized as a product of reductive metabolism. Respiratory tissue has been selected for this evaluation because of potential for exposure to 1-NP associated with diesel particles. *In vivo* studies have shown that inhaled diesel particles are readily deposited in the respiratory tract (*20,21*) in particular, in the alveolar macrophages (*21,22*). Although many of the particles are rapidly cleared from the lung, a substantial portion remain in the lung for a long period (*20,22*), thus increasing exposure to compounds associated with the particles. It has also been shown that both 1-NP and mutagenic activity were lost from diesel particles after incubation with pulmonary alveolar macrophages *in vitro* (*23*). We have now used diesel particles vapor-coated with ^{14}C-labeled 1-NP to study *in vivo* the fate of 1-NP absorbed onto diesel particles and evaluate interactions with pulmonary macromolecules.

Materials and Methods

Chemicals

1-Nitro[4,5,9,10 C^{14}]pyrene (^{14}C-1NP; 60 mCi/mmole), 1-nitro[^{3}H]pyrene (^{3}H-1NP, 6 Ci/mmole) and unlabeled

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1-nitropyrene (1-NP) were synthesized by Midwest Research Institute (Kansas City, MO). Unlabeled 1-NP was added to ^{14}C-1NP to adjust the specific activity to 10 mCi/mmole. Diesel particles vapor-coated with ^{14}C-1NP were prepared at Battelle Columbus Laboratories (Columbus, OH), by vaporizing ^{14}C-1NP at 275°C onto a stream of aerosolized diesel particles. The recovered particles contained 380 ppm nitropyrene and 195,000 dpm/mg particle. All chemicals used in the isolation and preparation of DNA for adduct analysis were purchased as follows: chloroform (spectro grade) from Burdick and Jackson (Muskegon, MI); isoamyl alcohol from Fisher Scientific (Raleigh, NC); phenol (99+%) from Aldrich Chemical Co. (Milwaukee, WI); and enzymes for DNA hydrolysis from Sigma Chemical Co. (St. Louis, MO) and Worthington Biochemical Corp. (Freehold, NJ). All other materials were purchased at the highest available grade of purity.

In Vitro Tissue Preparation and Incubation

Rabbit alveolar macrophages, tracheas and lung tissue were obtained from New Zealand White male rabbits (1.5–2.0 kg; Dutchland Laboratory Animals, Denver, PA) following lung lavage (*24*) and perfusion as described previously (*19*).

Macrophages (0.5×10^6 cells/mL), lung tissue (31.0 ± 4.0 mg/mL), or tracheal tissue (14.0 ± 0.4 mg/mL) were each incubated separately with ^{14}C-1NP (2 μg/mL) at 37°C in a humidified atmosphere of 5% CO_2 and air for 20 hr. The culture medium (M-199) contained 10% heat-deactivated fetal calf serum (FCS), penicillin (100 units/mL), streptomycin (100 μg/mL), kanamycin (100 μg/mL), and ^{14}C-1NP (8.1 μM; 81.0 nCi/mL) dissolved in DMSO (final concentration 0.5%, Burdick and Jackson Laboratories, Inc., Muskegon, MI). Macrophages (0.5×10^6 cells/mL), lung tissue (29.0 ± 2.0 mg/mL), and tracheal tissue (14.0 ± 1.0 mg/ml) were also cocultured under the same exposure conditions. Each experiment was repeated three times.

After incubation, the attached macrophages were detached by trypsinization and recovered by centrifugation at 300*g*, then lysed with 25 mL lysing buffer (50 mM Tris-HCl, 10 mM EDTA, 1% SDS, pH 8.0). The lung or tracheal tissues were separated from the culture medium by filtration through nylon mesh, then homogenized in 20 mL of the lysing buffer. A full description of these procedures has been given by King et al. (*19*).

Lung tissue (29.0 ± 2.0 μg/mL) was also incubated with ^{3}H-1NP (2 μg/mL) as described above to provide modified DNA of higher specific radioactivity for identification of the adducts formed.

In Vivo Studies

Male rats (300 g; Sprague-Dawley, Charles River CD-1, Wilmington, MO) received ^{14}C-NP-coated diesel particles by intratracheal instillation of 5 mg of particles in 0.2 mL phosphate-buffered saline with 1% Tween 80 under light halothane anesthesia. The animals were maintained individually in metabolism cages (Nalgene Co., Rochester, NY) for collection of urine and feces and received food and water *ad libitum*.

After 24 hr each animal was anesthetized with ether and the thoracic cavity opened to expose the lungs. The lungs were removed *en bloc* following total exsanguination of the animal. All vascular and connective tissues were removed and the lung tissue was placed into a 50-mL tube on ice. The tissue was thoroughly minced, than an aliquot (1 g) was weighed out and homogenized in 10 mL lysing buffer.

Quantitation of DNA and Protein Binding

DNA and protein were isolated according to an extraction scheme previously described (*25*) and illustrated in Figure 1. Briefly, protein was extracted from cellular nucleic acid in the lysates or homogenates with chloroform:isoamyl alcohol:phenol (CIP, 24:1:25) and precipitated with acetone. DNA was removed from the aqueous portion by precipitation with cold ethanol. Residual protein and RNA were removed by incubation with proteinase K (1 mg/mL) and NaOH, respectively. The radioactivity associated with the DNA was determined by liquid scintillation counting after acid precipitation onto glass fiber filters. Radioactivity associated with protein was determined by liquid scintillation counting of NaOH digested protein neutralized with HCl. Total DNA content was measured by the diphenylamine colorimetric assay (*26*) and total protein by the Lowry method (*27*).

Identification of DNA Adducts

The isolated DNA was redissolved in water and precipitated with ethanol in presence of sodium acetate until at least 95% of the radioactivity was precipitable. The DNA (<1 mg/mL) was dissolved in 10 mM Tris-HCl–0.1 M NaCl–5 mM $MgCl_2$ (pH 7.9) and was treated consecutively with 200 units of DNase I for 2 hr, 5 units of alkaline phosphatase for 2 hr, 2 units of phosphodiesterase I for 4 hr, 2 units phosphodiesterase II for 2 hr, and 5 units of alkaline phosphatase for 2 hr.

The resulting deoxyribonucleosides were then separated on a Sephadex LH-20 column. After application of the sample, the column was washed with water to remove unmodified deoxyribonucleosides. The modified adducts were then eluted with methanol and the solvent evaporated under reduced pressure. The sample was dissolved in 10 μL of methanol containing unlabeled 1-nitropyrene-modified DNA adducts [prepared according to Howard et al. (*1*)] and was analyzed by HPLC on a Dupont Instruments Model 850 high-pressure liquid chromatograph fitted with a C_{18}-μBondapak column (Waters Associates) using a concave gradient (number 2) from 50 to 100% methanol in water at 50°C and a flow rate of 1 mL/min. The eluate was monitored

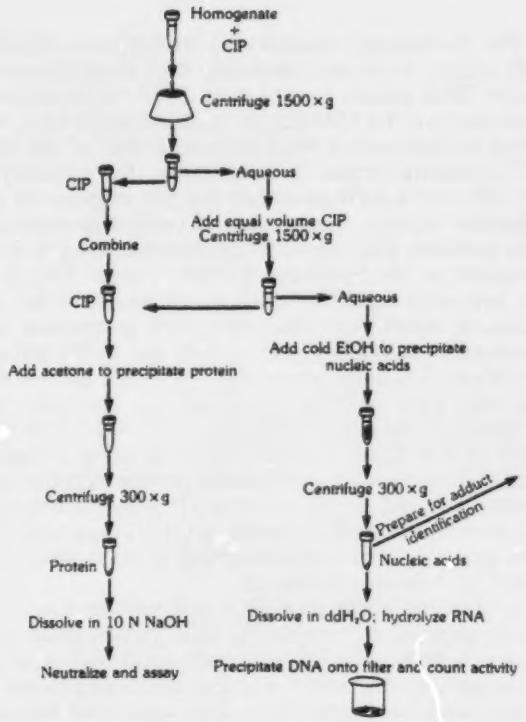

FIGURE 1. DNA isolation scheme. Protein was removed from cellular nucleic acid by two extractions of the cell lysates or tissue homogenates with chloroform:isoamyl alcohol:phenol (CIP, 24:1:25). Protein in the CIP portion was precipitated with acetone and DNA in the aqueous portion was precipitated with cold ethanol. After residual protein and RNA were hydrolyzed with proteinase K and NaOH, respectively, DNA was acid precipitated onto glass fiber filters and associated radioactivity measured in a liquid scintillation spectrometer. Samples used for identification of DNA adducts were not taken through the hyrolysis procedure, but were prepared directly as described in "Materials and Methods."

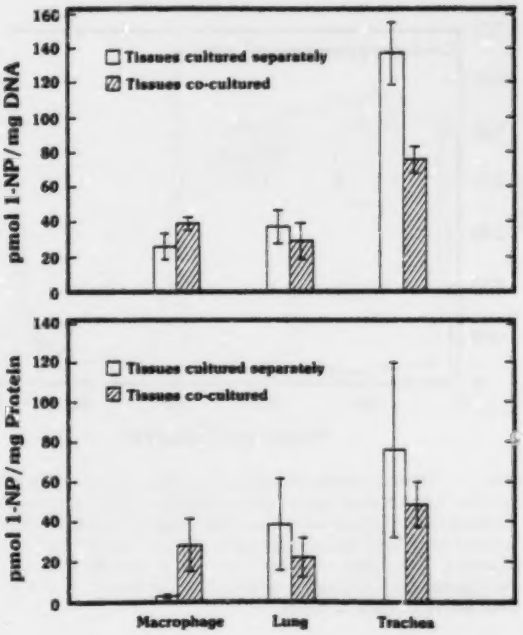

FIGURE 2. Binding of 1-nitro[^{14}C]pyrene metabolites to DNA and protein. Cultures of rabbit alveolar macrophages (0.5×10^6 cells/mL), lung tissue 31.0 mg/mL), or tracheal tissue (14.0 mg/mL) were incubated 20 hr in tissue culture media containing ^{14}C-1NP (8.1 μM; 81.0 nCi/mL). Cellular DNA (*A*) and protein (*B*) were isolated for analysis of binding as previously described. Similarly, rabbit alveolar macrophages (0.5×10^6 cells/mL), lung tissue 29.0 mg/mL) and tracheal tissue (14.0 mg/mL) were co-cultured 20 hr in media containing ^{14}C-1NP (8.1 μM; 81.0 nCi/mL). The cells and tissues were separated from one another as described and prepared for analysis of DNA and protein binding. Data are presented as the mean of three experiments ± standard error of the mean.

at 254 nm, and 1-min fractions were collected then assayed for radioactivity by liquid scintillation counting.

Results

Binding levels of ^{14}C-1NP metabolites to DNA and protein from respiratory tissues exposed *in vivo* are presented in Figure 2. Tracheal tissue exhibited the highest binding to DNA (Fig. 2*A*) and protein (Fig. 2*B*) in both the separate 20-hr cultures of isolated macrophages and lung and tracheal tissue and the cocultured system.

DNA from lung tissue incubated for 20 hr with ^{3}H-1NP was hydrolyzed and the resulting deoxyribonucleosides prepared for identification of modified adducts by high-pressure liquid chromatography as described in "Materials and Methods." A chromatogram from this analysis is shown in Figure 3. From 2 to 5% of the total DNA adducts co-eluted with the synthetic C8-dG-AP adduct in fraction number 60 (retention time 60 min). However, the majority (over 90%) of the modified DNA was not retained on the Sephadex LH-20 column and therefore was not amenable to HPLC analysis. Of the material retained on the LH20 column, 30% or more eluted with the solvent front, and the other two peaks observed (35 and 94 min) did not correspond to any synthetic standard currently available.

^{14}C-1NP vapor-coated onto diesel particles was administered to rats by intratracheal instillation to investigate the binding of 1-NP to respiratory tissue *in vivo* in an attempt to more closely model realistic exposure routes. Results are presented in Figure 4. After 24 hr, 6–8% of the total ^{14}C dose as well as a major portion of the particles (based on visual inspection) remained in the lungs. Analysis of DNA and protein binding in the lung tissue showed that 5 to 13% of the residual pulmonary ^{14}C (representing 0.6% of the total dose) was bound to protein, and no detectable ^{14}C was bound to DNA.

Discussion

Although macrophages and respiratory tissue are known to be able to metabolize PAH to reactive inter-

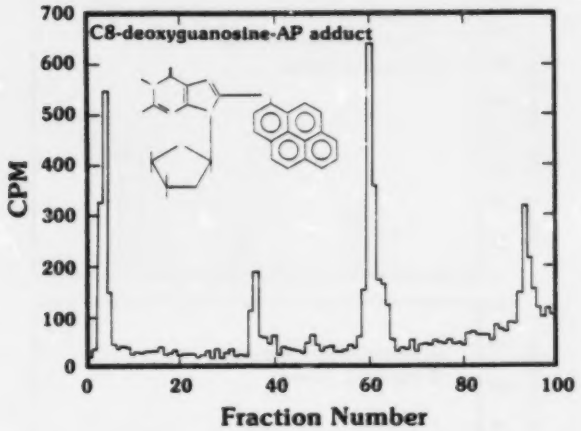

FIGURE 3. Chromatogram of lung DNA adducts from [H^3]1-nitropyrene. This chromatogram represents the HPLC profile of the modified deoxyribonucleosides obtained from the hydrolysis of DNA isolated from lung tissue exposed *in vitro* to ^{3}H-1NP (8.1 μM; 48 μCi/mL). The major peak (fraction 60) is identified as the C8-deoxyguanosine-1-aminopyrene adduct and represents 2–5% of the total activity bound to DNA.

mediates that bind DNA (*28*), the metabolism and binding of NO_2-PAH have only recently begun to receive attention. We have previously presented data that clearly show that macrophages and tissues from the respiratory tract are capable of binding 1-NP or its metabolites to DNA and protein (*19*). We have now identified one adduct bound to DNA from lung tissue exposed to ^{3}H-1NP.

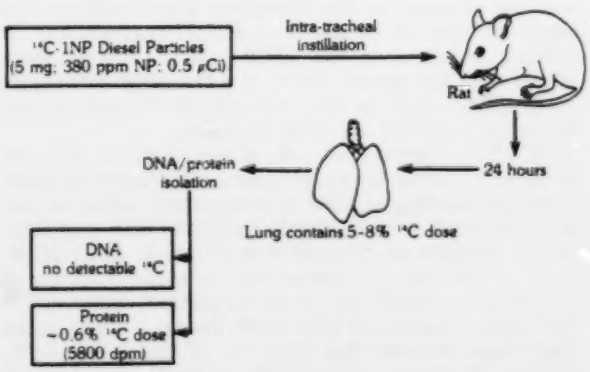

FIGURE 4. Disposition of ^{14}C from 1-nitro[^{14}C]pyrene vapor-coated onto diesel particles. Rats were treated with ^{14}C-1NP vapor-coated onto diesel particles by intratracheal instillation with the particles. Animals were maintained for 24 hr after dosing, then the lungs were removed and analyzed for total ^{14}C content. DNA and protein were isolated and ^{14}C bound to these macromolecules was measured as described in "Materials and Methods."

The *N*-(deoxyguanosin-8-yl)-1-aminopyrene (C8-dG-AP) adduct, which had previously been identified as the major DNA adduct formed from 1-NP by *Salmonella typhimurium* TA 1538 and by xanthine oxidase (*1*), was found to represent a small portion (2–5%) of the total DNA adducts formed by lung tissue. The majority of the NP–DNA adducts either did not respond to the digestive enzyme treatments, or yielded mononucleoside products that were not hydrophobic enough to be retained on the Sephadex LH-20 column. This is in marked contrast to the xanthine oxidase-catalyzed adducts, of which more than 80% were extractable and resolved into the C8-dG-AP adduct, and to the adducts formed in TA 1538, where the C8-dG-AP adduct was the only peak detected by HPLC (*1*). However, the presence of even small quantities of C8-dG-AP in mixtures of NP–DNA adducts from lung tissue is significant, in that it indicates that some portion of pulmonary metabolism and activation of 1-NP is by reduction of the nitro function, thus forming a C8-dG type of adduct that appears to be a characteristic of arylamines activated by *N*-hydroxylation (*1*).

Analysis of binding to DNA and protein from lung tissue exposed *in vivo* to ^{14}C-1NP vapor-coated onto diesel particles showed that a very small portion of the total radioactivity instilled in the lungs was bound to protein and that there was no detectable DNA binding. This is significant, however, because less than 10% of the total activity administered remained in the lung tissue even though most of the particulate matter was still present. This indicates that not only is respiratory tissue able to metabolize 1-NP, but that it is capable of removing it from diesel particles *in vivo*, as had previously been demonstrated for pulmonary alveolar macrophages *in vitro* (*23*). The majority of NP originally present on the diesel particles is therefore fully available for distribution throughout the body and for further metabolism and possible activation.

The information we have presented here strongly suggests that additional studies need to be conducted on the metabolism and binding of the NO_2 PAH found in diesel exhaust and urban air. More information is needed for defining the genotoxic burden of NO_2 PAH in ambient air. Further studies are currently being conducted in our laboratories to elucidate better the DNA and protein binding of 1-NP to tracheal epithelial cells. In addition, new DNA hydrolysis techniques are being evaluated to increase total DNA adduct recovery and identification.

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